Extracellular Cyclic AMP–Phosphodiesterase Accelerates Differentiation in *Dictyostelium discoideum*

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SUMMARY

Extracellular cyclic AMP–phosphodiesterase accelerates the development of aggregation competence in *Dictyostelium discoideum* when present during the pre-aggregation stage. The effect on development appears to depend only on hydrolysis of extracellular cyclic AMP and not on other properties of the phosphodiesterase molecule. Extracellular cyclic AMP–phosphodiesterase, as a promoter of differentiation, acts mainly throughout the first half of interphase. Our evidence supports the proposal that cyclic AMP oscillations control the rate and possibly the initiation of development. Since extracellular cyclic AMP–phosphodiesterase acts from the beginning of interphase cyclic AMP oscillations may also occur from early interphase, at least in the presence of this enzyme. This would imply that the cyclic AMP oscillator is a determinant, but not a product, of the developmental programme.

INTRODUCTION

Unicellular amoebae of *Dictyostelium discoideum* aggregate to produce a multicellular organism when food becomes scarce. The morphological and biochemical changes which accompany this process have been extensively studied since it affords a simple model system for the study of differentiation (Raper, 1950; Gerisch, 1968; Newell, 1971; Ashworth, 1971).

During aggregation the amoebae move toward each other in response to a chemical attractant which is almost certainly cyclic AMP (c-AMP) (Konijn *et al.*, 1968; Barkley, 1969; Roos *et al.*, 1975). Some of the steps in the developmental programme therefore concern the production and detection of c-AMP: the number of binding sites for c-AMP increases (Bonner *et al.*, 1969; Malchow & Gerisch, 1974) and cells develop the ability to relay signals, i.e. to emit a pulse of c-AMP, after detecting one from a neighbouring cell (Shaffer, 1957; Gerisch, 1968; Robertson, Drage & Cohen, 1972). Release of c-AMP (Malkinson & Ashworth, 1973) and of a rate substance inducing chemotaxis (Bonner, Kelso & Gillmor, 1966) occurs in interphase although spontaneous oscillations in extracellular c-AMP concentrations have been detected only in the late pre-aggregation stage (Gerisch & Hess, 1974; Roos *et al.*, 1975). The activity of membrane-bound c-AMP–phosphodiesterase (PDE) increases during pre-aggregation and aggregation; the activity of the extracellular form may steadily increase during the same periods or may form an early peak (at t_2) after interphase has continued for 2 h depending on the conditions of development (Riedel & Gerisch, 1971; Malchow *et al.*, 1972; Malkinson & Ashworth, 1973). In addition to the development of an autonomous chemotactic system, cells acquire aggregation-specific contact sites at their surface which are involved in stream formation (Beug, Katz & Gerisch, 1973*a*; Beug *et al.*, 1973*b*) and they become more adhesive (Bonner *et al.*, 1969). All these changes bring the

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cells to the developmental stage known as aggregation competence. This stage is defined operationally: cells are aggregation competent if they spontaneously form centres of aggregation and streams as soon as they are spread on a suitable surface.

In studies of signal propagation during aggregation, the duration of the interphase stage (t_{agg}) depended on cell density: at 5×10^5 cells/cm², aggregation began after 6 h, whereas at 5×10^4 cells/cm² it began after 13 h (Alcântara & Monk, 1974; see also Konijn & Raper, 1961; Konijn, 1968). It was also found (F. Alcântara, unpublished observations) that cells spending 9 h of interphase at low density were unable to respond to artificial sources of c-AMP. Thus even before aggregation and signal relay had started, the progress of development appeared to depend on some sort of cellular interaction.

One hypothesis to account for the dependence of t_{agg} on cell density is that accumulation of extracellular factors in the surrounding medium is either required for or stimulates the development of aggregation competence; at low cell densities such factors would accumulate slowly and aggregation would be delayed. In experiments to test this hypothesis, we found that an 'acceleration factor' (AF) was secreted into the medium during interphase. This factor accelerated the development of aggregation competence when added to cells at the beginning of interphase but when added later the effect was less marked. On DEAE chromatography, gel filtration and electrophoresis, the acceleration factor behaved as if it was identical with extracellular c-AMP-phosphodiesterase or very closely associated with it.

METHODS

Organism. *Dictyostelium discoideum* strain AX2 was kindly provided by Professor J. Ashworth.

Media. KK2 buffer contained (g/l water): KH₂PO₄, 2.25; K₂HPO₄, 0.67; MgSO₄·7H₂O, 0.05; pH 6.1. Axenic broth HL5 (Watts & Ashworth, 1970) contained (g/l water): bacteriological peptone (Oxoid), 14.3; yeast extract (Difco), 7.15; NaH₂PO₄·12 H₂O, 1.28; KH₂PO₄, 0.49; D-glucose, 15.4; pH 6.7. Non-nutrient (NN) agar contained 10 g Bacto-agar (Difco) and 1 l, KK2 buffer. Imidazole-HCl buffer contained (g/l water): imidazole, 1.37; Mg(CH₃COO)₂·4H₂O, 0.54; CaCl₂, 0.06; pH 7.0. Imidazole agar contained 10 g Bacto-agar (Difco) and 1 l imidazole-HCl buffer.

Optical method. A Wild M 20 microscope equipped with a humidity chamber was used for phase-contrast observations (magnification $\times 125$).

Growth conditions and initiation of development. AX2 cells were grown in HL5 medium at 22 °C on a rotary shaker (160 rev./min). Exponential-phase cells were harvested by centrifugation at 260 g for 1 min, washed twice in cold KK2 buffer and resuspended either in cold KK2 buffer or in cold test solution (see Results). Initiation of interphase (t_0) corresponds to the time of final resuspension, which followed harvesting by no more than 15 min. Aggregation competence was developed in the light at 22 °C in cells either agitated in suspension or spread on the surface of NN agar (5 ml NN agar freshly poured in 5 $\times$ 8 cm plastic boxes). NN-agar plates were incubated in humidity chambers provided with glass covers.

Preparation of AF solutions. AX2 cells were suspended in KK2 buffer for development in suspension. If not stated otherwise, cell density was 4×10^7 to 8×10^7 /ml. The extracellular medium was collected by centrifugation at 260 g for 1 min followed by another centrifugation at 10 000 g for 20 min, both at 4 °C. The time of collection (in hours) is indicated by the subscript of t : thus, for example, t_2 means that cells were collected after interphase had continued for 2 h, t_0 that cells were collected at the very beginning of interphase as soon as they had been resuspended, and t_{agg} that cells were collected at the end of interphase, at the onset of aggregation; t_{agg} is also a measure of the total duration of interphase.

When required, the supernatants were concentrated at 4 °C in a Millipore pressure filtration apparatus using a PSCA membrane (nominal retentivity 1000 daltons). These AF solutions were dispensed in small volumes and stored at -70 °C.

AF assay. When not stated otherwise, freshly harvested cells were suspended in test and control solutions at 5×10^6 to 7×10^6 cells/ml. Five 10 μ l replicate samples were taken from each suspension and deposited on freshly poured NN-agar plates. In the assay for rat-brain PDE, imidazole agar was used as substratum instead of KK2 agar. Incubation was according to the conditions described for development. The appearance of the first signals of cell aggregation – the formation of 3 to 4 cell-streams – was monitored by regular microscopic observation. AF activity is represented by the mean value of t_{agg} (time of onset of aggregation) for each set of replicas in comparison with the mean t_{agg} for controls.

Coupling of AF to Sepharose. AF solutions were dialysed overnight, at 4 °C against coupling buffer (0.05 M-sodium borate buffer pH 8.0 containing 0.5 M-NaCl). CNBr-activated Sepharose-4B (1 g; Pharmacia) was washed in 1 mM-HCl and 0.5 g wet gel was used for coupling AF in each of three 3 ml portions of AF solution. Coupling was carried out at room temperature for 5½ h and then at 4 °C overnight. The gels were then washed in coupling buffer and resuspended for 2 h in 1 M-ethanolamine-HCl pH 8.0, followed by six rinsing cycles, first with 0.1 M-sodium acetate buffer pH 4.0 containing 1 M-NaCl and then with 0.1 M-borate buffer pH 8.0 also containing 1 M-NaCl. The gels were washed, resuspended in KK2 buffer and stored at 4 °C after addition of 0.02% (w/v) NaN_3 . A control gel was prepared identically, but omitting the AF coupling step. AF and PDE assays were done with freshly washed gels. For AF assays, 10 μ l drops of a suitable gel dilution were deposited on NN-agar plates, allowed to settle and the supernatant removed by suction; cell suspension in KK2 buffer (10 μ l) was then placed on the gel layer. For PDE assays the gel was kept in suspension by agitation during the reaction.

Fractionation of AF solution on DEAE cellulose. A six-times concentrated t_{10} AF solution (55 ml) was dialysed overnight against 20 mM-triethanolamine-HCl (TEA-HCl), pH 6.8, and applied to a column (25 $\times$ 2 cm) of Whatman DE 52 cellulose equilibrated with the same buffer. The column was first washed with 50 ml TEA-HCl buffer and then eluted with a gradient of 0.0 to 0.4 M-KCl in 20 mM-TEA-HCl, pH 6.8. These procedures took place at 4 °C. Fifty 4 ml fractions were collected and assayed for AF and PDE activities. E_{280} was measured in a Beckman spectrophotometer.

Filtration on Sephadex G200. Following fractionation of an AF solution on DE 52 cellulose, 0.5 ml portions of each fraction within the coincident PDE and AF peaks (fractions 35 to 39) were pooled (total volume 2.5 ml) and applied to a column (55 $\times$ 1.5 cm) of Sephadex G200, with Blue Dextran 2000 and NaN_3 as markers. Elution was carried out at 4 °C with 0.017 M-sodium-potassium phosphate buffer pH 6.1. Each 1.2 ml fraction was assayed for PDE and AF activities and E_{280} measured.

Disc gel electrophoresis. This was carried out in tris-glycine, pH 8.3/tris-HCl, pH 8.8, polyacrylamide gels (7%, w/v), according to Davis (1964). The supernatant from t_{10} cells was concentrated 93 times by pressure filtration on Millipore PSCA filters and dialysed overnight against 50 mM-tris-HCl, pH 6.7. Samples (100 μ l) containing 340 μ g protein were applied to 0.5 $\times$ 11 cm gels together with a bromophenol blue marker. Electrophoresis, at 4 °C, was at 0.5 mA/gel for 20 min and then at 3 mA/gel. When the tracking dye neared the bottom (after approximately 2 h) the gels were removed and either frozen at -70 °C or fixed and stained. For PDE and AF assays the frozen gels were sliced in 1 mm sections; each section was eluted overnight in 0.5 ml of 10 mM-TEA-HCl, pH 6.8.

Rat-brain PDE. Three rat brains were homogenized and fractionated according to

Thompson & Appleman (1971). After gel filtration on Bio-Rad A 1·5, fractions in the second (later eluting) peak of PDE activity were combined and concentrated ten times by pressure filtration on Diaflo (Amicon) UM2 membranes (retentivity ≥ 1000 daltons). The concentrate was dialysed at 4 °C against 500 ml imidazole-HCl buffer for 5 h with a change of buffer at 2·5 h. The dialysate was clarified by centrifugation at 12000 *g* for 10 min and kept at 4 °C (freezing inactivated the enzyme). The PDE activity obtained was 50 units/ml and the K_m value was approximately 1 μ M-c-AMP (data not shown).

PDE assay. PDE solution (100 μ l) at appropriate dilution was incubated at 35 °C with c-AMP (approximately 60 μ M) in 1·0 ml 0·02 M-TEA-HCl, pH 7·4, containing 7 mM-MgCl₂, 10 μ g alkaline phosphatase (350 u/mg) and 10 μ g adenosine deaminase (200 u/mg) (Michal & Bergmeyer, 1970). The change of E_{265} , read on a Beckman DB-G spectrophotometer, was continuously recorded. One unit of PDE activity is defined here as the amount of PDE hydrolysing 1 nmol c-AMP/min at 35 °C.

Alkaline phosphatase and adenosine deaminase were purchased from Boehringer Mannheim, Germany.¹

PDE inhibitor. A preparation of extracellular PDE inhibitor was obtained by heating 50 ml of AX2 t_{14} supernatant to 80 °C for 10 min and then adding ammonium sulphate to 90% saturation (Riedel *et al.*, 1972). The precipitate was dissolved in 5 ml of 10 mM-TEA-HCl, pH 6·8, dialysed overnight against the same buffer and stored frozen at -70 °C. Purified inhibitor was prepared by DE 52 cellulose chromatography of unheated, dialysed supernatant. The fractions with high inhibitor activity were combined and frozen at -70 °C.

PDE inhibition. This was carried out according to Riedel & Gerisch (1971).

Protein content. This was determined according to the method of Lowry *et al.* (1951).

RESULTS

Dependence of t_{agg} on cell density

Alcântara & Monk (1974) reported the dependence of t_{agg} on cell density in populations developing in KK2 buffer. Figure 1 (broken lines) shows the results from three sets of experiments on the variation of t_{agg} with cell density, in cells suspended at t_0 in KK2 buffer. Above 10^7 cells/ml the variation of t_{agg} with density was not significant, but at lower densities t_{agg} increased steadily from about 4½ h to 14 h.

Time of onset of aggregation in AF-treated cells

The extracellular medium from aggregation-competent cell suspensions was collected (see preparation of AF solutions). Freshly harvested AX2 cells were suspended in this medium at from 3×10^5 to 10^9 cells/ml and t_{agg} was determined by microscopic observation (see Methods). Figure 1 (full lines) shows the results of these experiments. Parallel experiments carried out with the same stock cells suspended in KK2 buffer gave results which are also represented in Fig. 1 (broken lines).

The difference in duration of interphase between the KK2-treated and the AF-treated cells increased progressively with decreasing cell densities from 1 to 8 h. This demonstrated that interphase may be accelerated by a factor(s) present in the extracellular medium of aggregation-competent cultures, now designated as AF solutions.

In the presence of AF and at 10^7 cells/ml or more, the t_{agg} value was minimal; at less than 10^7 cells/ml t_{agg} increased steadily with decreasing cell density. The assays with different sample volumes (10 and 5 μ l) were meant to test the effect of small errors in the volume taken for AF assays. The results showed that at the cell density selected for the standard

Fig. 1. Dependence of t_{agg} on cell density. Results of three experiments ($\circ$, $\bullet$; $\square$, $\blacksquare$; ∇ , $\blacktriangledown$) are given. Development was initiated in AX2 cells suspended in either KK2 buffer (---) or AF solution (—). After adjustment to the desired initial cell density both cell suspensions were successively diluted in the appropriate medium. From the series of cell suspensions samples of $10\ \mu\text{l}$ (open symbols) and $5\ \mu\text{l}$ (closed symbols) were placed on NN-agar plates and incubated at 22°C with illumination. The time of onset of aggregation (t_{agg}) was monitored microscopically; points represent the mean t_{agg} for each set of five replicas (deviation from the mean ≤ 15 min).

assay (5×10^6 to 7×10^6 cells/ml) small variations in volume and consequently in cell number did not affect t_{agg} . Comparison of populations with the same total number of cells but in which cell density was varied by a factor of 2, showed that, with populations greater than 5×10^3 cells, it was cell density and not cell number which affected the duration of interphase; below this value both factors appeared to be relevant.

Although cell density proved to be critical in the AF assay, it was not the only factor controlling t_{agg} . The same strain, harvested in the exponential phase of growth (see Gerisch *et al.*, 1975a) may on different occasions display different rates of development. This was reflected in variations of several hours in the value of t_{agg} in separate experiments, but the response to the presence of AF was always marked.

Partial purification of AF

In the experiments described above we found that an acceleration factor is present in the suspension medium of aggregation-competent cells. This factor accelerated the development

Fig. 2. DEAE cellulose chromatography of a t_{10} supernatant. The column was eluted with a continuous gradient of 0.0 to 0.4 M-KCl in 10 mM-TEA-HCl, pH 6.8. AF activity, expressed as t_{agg} , was measured in two experiments (∇ , $\blacktriangledown$); (t_{agg} values for the KK2 controls are shown on the ordinate); $\bullet$, PDE activity; —, E_{280} . Deviation from the mean $t_{agg} \leq 15$ min.

of aggregation competence when it was added at the beginning of the period of starvation. To investigate the nature of the factor(s) involved, we purified AF solutions by DEAE cellulose chromatography, gel filtration and electrophoresis. We always obtained a single peak of AF activity, the acceleration factor behaving as if it was identical with c-AMP-phosphodiesterase or very closely associated with it.

Fractionation on DEAE cellulose. AF was purified from a six-times concentrated t_{10} AF solution by chromatography on DEAE cellulose. Figure 2 shows that AF activity and one peak of PDE activity co-fractionated when eluted with a gradient of 0.0 to 0.4 M-KCl in 10 mM-TEA-HCl at pH 6.8. The first PDE peak (fractions 22 to 34), devoid of AF activity, was found to correspond to the high K_m form of extracellular PDE; the second peak, coincident with AF activity, had a K_m of approximately 10^{-6} M-c-AMP (data not shown). The specific activity of extracellular PDE in fraction 37 was 360 u/mg protein, compared with 36 u/mg protein in the initial AF solution.

Fractionation on Sephadex G200. After DEAE cellulose chromatography, fractions 35 to 39 were combined and eluted from a Sephadex G200 column with 0.017 M-sodium-potassium phosphate pH 6.1. Figure 3 shows the elution profiles of AF and PDE activities with coinciding peaks between fractions 18 and 30. PDE in fraction 23 had a specific activity of 776 u/mg protein. This represents a 2-fold purification in relation to the enzyme in fraction 37 of DEAE cellulose chromatography (Fig. 2). The void volume for this column corresponded to the first 18 fractions (22 ml). The end marker (NaN_3) was eluted at fraction 60 (72 ml).

Disc gel electrophoresis. After electrophoresis of a 93-times concentrated AF solution on 7% polyacrylamide gels, 16 protein bands were revealed on staining with Coomassie blue. The migration distances ranged from 0.4 to 8.5 cm from the origin (bromophenol marker at 10.3 cm). AF activity, coinciding with the larger peak in PDE activity (Fig. 4), was maximal

Fig. 3. AF and extracellular PDE purification on a Sephadex G200 column (55×1.5 cm) after DEAE cellulose chromatography. Fractions (1.2 ml) were collected during elution with 0.017 M-sodium-potassium phosphate buffer, pH 6.1. Blue-dextran 2000 eluted in fraction 18 (void volume, 22 ml) and the end marker (NaN_3) in fraction 60 (72 ml). (▼), AF activity, expressed as mean t_{agg} for three replicas, 15 min (t_{agg} for the KK2 control is indicated on the ordinate); ●, PDE activity; —, E_{280} . Deviation from the mean $t_{agg} \leq 15$ min.

Fig. 4. Disc electrophoresis of AF solution (93 times concentrated t_{10} supernatant) on 7% polyacrylamide gel. ●, PDE activity; ▼, AF activity, expressed as mean t_{agg} for three replicas (the point on the ordinate represents t_{agg} in the KK2 control). Deviation from the mean $t_{agg} \leq 30$ min.

at 1.4 cm where no dense protein band was observed in stained replicas. PDE activity formed a second peak into a region of high protein content at 1.7 cm from origin; probably this peak corresponded to the high K_m form of extracellular PDE.

AF activity of rat-brain PDE

On the basis of the evidence presented above we tested the hypothesis that PDE activity could account for AF activity, by using a completely distinct PDE with a K_m value similar to that of *D. discoideum* PDE (approximately 10^{-6} M-c-AMP).

Fig. 5. AF and PDE activities in extracellular medium during interphase. Exponentially growing AX2 cells were resuspended in KK2 buffer at (cells/ml): ($\circ$, $\bullet$) 8×10^7 ; ($\square$, $\blacksquare$) 4×10^7 ; ($\blacktriangledown$) 3×10^6 . The cells were either suspended by agitation (a, c) or spread on NN agar plates (b, d). Samples of extracellular medium were collected at various times after commencement of interphase, stored as AF solutions and assayed for PDE activity (a, b) or AF activity expressed in terms of t_{agg} (c, d). Each point in (c) and (d) represents the mean t_{agg} for five replicas; variation from the mean was ≤ 15 min.

Table 1. AF activity of rat-brain PDE

Assays were done on imidazole agar, with AX2 cells suspended in rat-brain PDE solutions or in control solutions (AX2 AF solution, KK2 buffer and imidazole buffer). The final PDE activity in each solution (units/ml) is given in parenthesis. Results are the means of five replicas; deviation from the mean did not exceed 15 min. Each line corresponds to a separate experiment.

Mean aggregation time, t_{agg} (h)			
Rat-brain PDE 7(8)	AX2 AF solution 7(12)	KK2 buffer 9½ (o)	Imidazole buffer 10(o)
5(50)–6(20)	5(18)	8(o)	8(o)
6(4)–6(20)	5(9)	8(o)	8(o)
6(4)–6(20)	6(9)	8(o)	8(o)

Rat-brain PDE was prepared according to Thompson & Appleman (1971). AF assays were carried out on imidazole agar both for test and control solutions, as rat-brain PDE activity appears to require Ca^{2+} and Mg^{2+} and is enhanced by imidazole (Cheung, 1967; Lin, Liu & Cheung, 1974).

Table 1 shows that rat-brain PDE stimulates acceleration of interphase to a degree comparable to AF from AX2; this indicates that the AF effect depends only on c-AMP hydrolysis and that interactions of AX2 PDE with other molecules are not involved.

Fig. 6. Plots of t_{agg} versus extracellular PDE activity. (a) Series of crude AX2 t_2 supernatants at various dilutions (6 experiments); (b) series of extracellular PDE, inhibited to various degrees. The supernatant was diluted in KK2 buffer; the ammonium sulphate preparation of a heated t_{14} supernatant was used as the source of PDE inhibitor. In one experiment ($\square$) the inhibitor solution was partially purified by DEAE cellulose chromatography. AF assays (expressed as t_{agg}) were performed in parallel for each pair of series with the same symbols. Each point represents the mean t_{agg} for each set of five replicas. Points on the ordinate represent the time of aggregation in the control (KK2 buffer). Maximum deviation from the mean $t_{agg} \leq 15$ min.

Time course of AF and extracellular PDE activity changes during interphase

To investigate further the identity of AF and extracellular PDE, we followed the kinetics of changes in both activities in the extracellular medium of populations in the pre-aggregation stage. Populations of cells at different densities were kept in suspension in KK2 buffer with agitation at 160 rev./min or were spread as a surface film on NN-agar plates. The suspensions were sampled at intervals and a cell-free supernatant was collected and treated as described for preparation of AF solutions. At 4×10^7 and 8×10^7 cells/ml extracellular PDE activity was maximal at t_2 (Fig. 5a) after which it decreased to about 2.5 u/ml. The peak of activity at t_2 failed to appear in more sparse populations (3×10^6 cells/ml) or when cells at the higher densities developed on agar surfaces (Fig. 5a, b). Although extracellular PDE formed a peak at t_2 in denser populations, AF activity could increase till t_5 (Fig. 5c). It appears that the later supernatant preparations with an extracellular PDE activity of about 2.5 u/ml are the most active in accelerating aggregation, while the apparent maximum level of extracellular PDE activity in earlier (t_2) supernatants may not provide maximum AF activity.

PDE activity versus AF activity

Results presented above (Fig. 5) have shown that AF solutions with low levels of PDE activity were able to decrease t_{agg} from 8 h (control) to 4 h; solutions with less than 2.5 u PDE activity/ml produced a less significant decrease. Within the error involved in the AF assay (± 0.25 h), data from six experiments (Fig. 6a) again show that 2.5 u/ml is the mean minimum value of PDE activity still compatible with maximum AF activity. Populations

with extended interphases, as shown by KK2 controls, seemed to require higher PDE activities at t_0 (approximately 5 u PDE/ml).

AF activity of inhibited PDE

Figure 5(a) and (c) shows that although extracellular PDE activity reached its peak at t_2 , when interphase took place in shaken suspensions, this peak did not coincide with maximum AF activity, which could occur 2 to 3 h later. At this time extracellular PDE activity had decreased significantly and it is known that this decrease results from the secretion into the medium of a natural inhibitor of extracellular PDE (Riedel & Gerisch, 1971; Riedel *et al.*, 1972, 1973). We therefore attempted to determine whether inhibited extracellular PDE still possesses AF activity. Inhibitor was prepared either by ammonium sulphate precipitation of a heated t_{14} supernatant or by partial purification of a fraction of the same supernatant on a DEAE cellulose column. The two methods did not produce significantly different results. The t_2 supernatants, at a constant final dilution, were used as PDE source and the degree of PDE inhibition was varied by the addition of a range of concentrations of inhibitor.

Partial inhibition of extracellular PDE to an activity below 2.5 u/ml decreased AF activity (Fig. 6b). Again, a PDE activity of 2.5 u/ml appears to be the lowest limit consentaneous with maximum AF activity. When extracellular PDE activity was added to solutions of partially-inactivated extracellular PDE, high levels of both AF and PDE activities were restored, in contrast to controls where KK2 buffer was substituted for the extra PDE solution. Loss of AF activity is therefore due to loss of PDE activity and not to a toxic effect of the PDE-inhibitor. The PDE-inhibitor solution alone, added at t_0 allows for a t_{agg} similar or slightly increased (by 1 to 2 h) in relation to the control. We conclude that inhibited extracellular PDE is not active as AF and that 2.5 u PDE/ml is, approximately, the threshold at which maximum AF activity results. We do not know why AX2 t_2 supernatants of agitated cell suspensions never showed AF activity as high as late supernatants; possibly t_2 supernatants contain a product which is slightly inhibitory for differentiation, or contain a higher proportion of high- K_m PDE than supernatants taken at later times.

AF activity of insolubilized AF

To investigate if AF activity was limited to the extracellular medium, as the results with rat-brain PDE seemed to indicate, we prevented its uptake by the cells or its incorporation at the cell surface by covalently binding AF to Sepharose beads. Insoluble AF was obtained from three separate preparations: crude t_{14} supernatant; crude t_2 supernatant; t_{14} supernatant purified by DEAE cellulose chromatography. Recovery of PDE activity after coupling to CNBr-activated Sepharose-4B varied between 1.5 and 3.6%. The final PDE activities in the different gel suspensions are indicated in Table 2. For AF assays, 10 μ l of gel suspension were placed on NN-agar plates and the excess liquid was sucked off before deposition of 10 μ l of a cell suspension in KK2 buffer on to the sedimented beads. Results in Table 2 show that in Expt A, where a high density of beads was used, the AF activity in soluble and insoluble AF was identical. In Expts B and C the gel suspensions were diluted by a factor of 2 to circumvent the difficulty in microscopic observation; t_{agg} in these preparations, although increased in relation to the soluble forms of AF, was still markedly less than the value for the KK2 control. Supernatants derived from AF gels and a control gel (inactivated Sepharose-4B) failed to produce any acceleration of interphase. It appears likely, therefore, that AF activity of PDE is due to c-AMP hydrolysis in the extracellular medium.

Table 2. *AF activity of insolubilized AF compared with activity in the corresponding soluble forms*

PDE activity (units/ml) is given in parentheses. Data from three experiments are presented. In expt (A), a high density of beads was used; in expts (B) and (C) the gel suspensions were diluted 2-fold in KK2 buffer, to facilitate microscopic observation. The supernatants of AF gels and a control gel (inactivated-Sepharose-4B) produced t_{agg} values similar to t_{agg} in the KK2 control.

	t_{agg} (h)		
	(A)	(B)	(C)
Insoluble t_{11}	6 (2.5)	5½ (1.25)	6 (1.25)
Soluble t_{11}	5½ (16)	3½ (16)	4½ (16)
Insoluble t_2	5½ (4)	5½ (2)	6 (2)
Soluble t_2	6 (64)	4 (64)	4½ (64)
Insoluble F t_{11} *	6½ (2.5)	5 (1.25)	5½ (1.25)
Soluble F t_{11} *	6 (20)	4 (20)	4½ (20)
KK2 buffer	8½ (0)	7½ (0)	9½ (0)

* AF in DEAE-fractionated t_{11} supernatant.

Effect of 5'-AMP, 5'-AMP, A and adenine on duration of interphase

Addition of 5'-AMP, adenosine or adenine, at 1 μ M or 0.5 mM, to the KK2 buffer used as suspending media in AF assays did not cause acceleration or retardation of interphase in comparison to the KK2 control. c-AMP (1 μ M) was equally ineffective but 0.5 mM-c-AMP caused a small delay in the onset of aggregation. In conditions where the AF solution promoted aggregation competence in 3.5 h, addition of 0.5 mM-c-AMP to AF increased t_{agg} to 5.5 h (t_{agg} in the KK2 control = 8 h). Adenine, adenosine and 5'-AMP at 0.5 mM in AF solution did not affect t_{agg} as compared to the AF control. These results support the conclusion that AF activity depends on the hydrolysis of extracellular c-AMP but not on the products of this hydrolysis.

Chemotaxis and stream formation in AF-treated cells

We wanted to know whether AF (as PDE) accelerated the onset of aggregation by facilitating signalling after the aggregation competence stage, or whether it actually increased the rate of differentiation toward this stage. If the effect was only to facilitate signalling, then chemotaxis toward artificial sources of c-AMP, which does not require cell signalling, might develop in AF-treated populations at the same time as in the control (KK2-treated) populations. On the other hand, if differentiation was accelerated, both chemotaxis and streaming (which includes increase in c-AMP receptors, capacity for signal amplification and formation of EDTA-resistant cell contacts) would develop earlier in AF-treated cells than in controls.

At the beginning of interphase, cells at 7×10^6 /ml were resuspended in AF solution or KK2 buffer and several 10 μ l samples were deposited on NN-agar plates. At hourly intervals during interphase, two samples of each population were challenged with 5 μ l of a 0.1 mM-c-AMP solution placed at about 1 mm from the periphery of each population. The times of positive chemotactic response as separate cells (detected as a common orientation in cell elongation within the c-AMP source range) and of stream formation towards the artificial source of attractant were determined by microscopic observation (Table 3); KK2 controls, to measure spontaneous aggregation, were not challenged with c-AMP but otherwise conditions were the same. Chemotaxis was never evident in the controls before t_8 but AF-

Table 3. *Response to an artificial source of c-AMP by AF- and KK2 buffer-treated cells*

At t_0 several 10 μ l samples of suspensions at 7×10^6 cells/ml of KK2 buffer or AF solution were deposited on NN-agar plates and incubated. Samples were tested hourly for response toward sources of c-AMP (10^{-4} M solution) placed approximately 1 mm from their periphery. Time of chemotactic response as separate cells and with formation of streams is indicated, as well as t_{agg} in non-challenged populations. In expts (A) and (B), cells were suspended in two times concentrated t_{14} supernatant (44 units/ml PDE); in expt (C), cells were suspended in t_2 supernatant (20 units/ml PDE).

Expt no.	Extracellular medium	Chemotaxis		Spontaneous aggregation t_{agg} (h)
		Separate cells (h)	Streams (h)	
A	AF	4	5½	6
	KK2	8	8-9	10
B	AF	4-5	5-6	6
	KK2	8-9	8-10	10½
C	AF	3½-5	5½	5½
	KK2	8-9	9	9½

treated cells showed a clear response from about t_4 or t_5 . The time when ability to form streams developed was always very close to the time of spontaneous aggregation (t_{agg}), though this differed in AF and control populations by 3.5 to 4 h. Results for 10 μ M-c-AMP solutions were similar, except that the influence of c-AMP had a smaller range. These results support the hypothesis that AF accelerates the rate of differentiation toward aggregation competence.

Period of action of AF

The results so far indicate that at some stage of interphase extracellular c-AMP inhibits development, but that the addition of extra PDE at this stage overcomes this barrier. It was important to delineate this period. We therefore investigated the effects of the addition and removal of AF at various times during interphase.

AX2 cells suspended in AF solutions or KK2 buffer at the initiation of interphase were either kept in suspension with agitation (Fig. 7*a, b*) or were spread as a film on the surface of NN-agar plates (Fig. 7*c, d*). Each cell preparation was sampled several times during interphase by taking 100 μ l volumes directly from the suspension or from the agar preparations. The latter was done after gentle detachment of the cells by re-spreading with a glass rod; distinct cell preparations were used each time to avoid repeated re-spreading. After centrifuging at 260 *g* for 1 min, cells from each sample were resuspended at the initial density (5×10^6 to 7×10^6 cells/ml) either in KK2 buffer or in freshly-thawed AF solution. At each time (see Fig. 7) four cell suspensions were obtained: AF pretreated cells resuspended in AF solution (control); AF pretreated cells resuspended in KK2 buffer (to determine the effect of difference in time of removal of AF); KK2 pretreated cells resuspended in KK2 (control); and KK2 pretreated cells resuspended in AF solution (to determine the effect of difference in the time of addition of AF). Ten μ l samples (five replicas) of each cell suspension were deposited on NN agar and t_{agg} was monitored by microscopic observation. At this cell density, the conditions of incubation during pretreatment (shaken suspensions or preparations on agar) did not appear to have any marked effect on the overall results. AF activity, measured as t_{agg} in relation to the KK2 control, was decreased (though to a progressively smaller extent) by a delay in the addition of AF, up or close to the stage of aggregation competence. This indicates that, besides its function in early interphase, AF may continue to

Fig. 7. Period of action of AF. Four experiments were conducted in which Ax2 cells, suspended at t_0 in KK2 buffer and AF solutions, were either kept in suspension by agitation (a, b) or spread on NN-agar plates (c, d). After interphase had continued for various times, samples were taken from the initial cell suspensions, and either (■) the AF solution was replaced by KK2 buffer, or (●) KK2 buffer was replaced by AF solution. Controls were under the same conditions except that cells were resuspended in the same type of medium as used for pretreatment: (○), AF-pretreated cells resuspended in AF; (□), KK2 buffer-pretreated cells resuspended in KK2, buffer. Time of aggregation (t_{agg}) was monitored microscopically on 10 μ l populations deposited on NN agar when resuspended. Each point represents the mean t_{agg} for each set of five replicas; deviation from the mean ≤ 15 min. Points enclosed in bars denote immediate re-aggregation. PDE activity in the AF solutions: (a, b) 10 units/ml; (c) 20 units/ml; (d) 13 units/ml.

act until late in the pre-aggregation stage. The effect of the presence of AF during the first hour of development was marked. Removal of AF at different times of interphase in three of the experiments indicated that, although active since t_0 , the effects of AF during the first hours of interphase were reversible. AF (extracellular PDE) became completely dispensable when cells reached the stage of aggregation competence, as shown by the immediate re-aggregation of competent cells suspended in AF or KK2 solutions (Fig. 7, points enclosed in bars).

DISCUSSION

Preliminary experiments showed that the AF was thermo-labile and was retained by ultrafiltration membranes with a nominal cut-off at 1000 daltons, which indicated that AF was probably not a small molecule such as a nucleotide. Present evidence suggests that it is a protein. It is precipitated by ammonium sulphate. On DEAE cellulose and gel filtration columns, fractions exhibiting AF activity also exhibited a proportionate PDE activity. Electrophoresis on polyacrylamide gels also revealed that the two activities coincided. The inference that PDE activity is necessary for AF to affect development is supported by the evidence that a specific inhibitor of extracellular PDE can diminish the effect of the AF (Fig. 6). Extracellular PDE may have a role during growth (Malchow, Fuchila & Nanjundiah, 1975), and during aggregation it may reduce the signal to noise ratio (Shaffer, 1956; Chassy, Love & Krichevsky, 1969; Goidl *et al.*, 1972) and steepen c-AMP gradients (Bonner *et al.*, 1969; Chassy, 1972; Gerisch *et al.*, 1972). The extracellular inhibitor of extracellular PDE has been studied by Gerisch and his collaborators (Riedel & Gerisch, 1971; Gerisch *et al.*, 1972; Riedel *et al.*, 1972, 1973). Its role is unknown, but it was suggested that inhibition of extracellular PDE may be necessary during later interphase so that the c-AMP concentration can rise to a level which maintains the shift from vegetative to developing state (Chassy *et al.*, 1972; Bonner, 1970; Riedel & Gerisch, 1971; Gerisch *et al.*, 1972). The limited data available might lead one to expect that extracellular PDE plays an active part in aggregation but not in interphase. We found, on the contrary, that it made little difference whether extracellular PDE was present during aggregation but that this did have a marked effect on the duration of interphase (Fig. 7). We are thus left without any role to ascribe to the slime-mould extracellular-PDE inhibitor.

The close association of AF and PDE activity throughout various fractionation procedures indicated that PDE alone, or PDE complexed with other proteins, might be responsible for the effects of AF. We therefore attempted to determine whether these effects were due solely to PDE activity or whether some function in addition to the hydrolysis of c-AMP is involved. For example, PDE might have a dual role, as an enzyme and as a participant in the formation or stabilization of cell contacts. The experiments with rat-brain PDE were carried out with the aim of testing this possibility. It is unlikely that rat-brain enzyme can form specific complexes with *D. discoideum* proteins as postulated for a 'dual role' slime-mould PDE. The only significant property the two enzymes are likely to have in common is the capacity to hydrolyse c-AMP. Therefore the ability of rat-brain PDE to reproduce the effects of slime-mould PDE on development during interphase is evidence that these effects depend only on the extracellular c-AMP concentration and not on any other factor, i.e. a dual role for slime-mould extracellular PDE seems to be excluded.

Evidence for the exclusively extracellular activity of AF is also provided by the experiments with Sepharose-bound slime-mould PDE, which, after binding, kept both activities.

Consistent with this interpretation of the effects of PDE in our experiments, we also found that c-AMP (but not 5'-AMP, A or adenine) was able to decrease the apparent AF activity when added at t_0 at a concentration of 0.5 mM. Moreover, none of the tested products of c-AMP hydrolysis (5'-AMP, A or adenine) at concentrations of 1 μ M or 0.5 mM had any effect on the duration of interphase. This suggests that t_{agg} is dependent on the extracellular c-AMP concentration.

To distinguish between possible early or late effects of PDE on the development of aggregation competence we followed the expression of the ability to respond chemotactically to an artificial source of c-AMP (Table 3). Increased chemotactic ability develops before

aggregation competence (Bonner *et al.*, 1969), so if extracellular PDE acted only during the process of aggregation itself, or at a stage immediately preceding it, then the development of chemotactic ability should be unaffected by PDE pretreatment. Alternatively, if extracellular PDE acted early in interphase, then chemotaxis, as well as aggregation competence, might be expressed earlier in PDE-treated cells than in controls. We found that the development of chemotaxis was accelerated by extracellular PDE. This, and the results of experiments in which cells were treated at various times throughout interphase with extracellular PDE (Fig. 7), suggest an early action of extracellular PDE (AF). It seems that there is a period (t_0 to t_2) during which there is high sensitivity to AF and its effects are reversible; at a later stage (t_2 to t_{agg}) the cells are less sensitive to AF and its effects are largely irreversible (Fig. 7).

The variation of t_{agg} with cell density might also be explained on the basis of PDE activity: we suggest that extracellular PDE (AF) becomes dispensable when the membrane-bound PDE activity reaches a threshold value at some time before aggregation. In dense populations, the combined activity of extracellular and membrane-bound phosphodiesterases reaches the threshold value early in interphase and so development is accelerated. In sparse populations, the extracellular c-AMP concentration is probably controlled locally by membrane-bound PDE, as extracellular PDE activity in the medium must be negligible.

The experiments with rat-brain PDE, slime-mould PDE immobilized on Sepharose and with inhibited slime-mould PDE all indicate that extracellular c-AMP concentration is a major factor in determining the duration of interphase. Possible explanations are: (i) low c-AMP is a developmental trigger which starts a chain of biochemical changes; (ii) low extracellular c-AMP accelerates development but by a continuous rather than a triggering mechanism; (iii) high c-AMP concentrations inhibit development and low c-AMP concentrations do not accelerate it. Hypothesis (i) may be discarded as cells briefly exposed to PDE in early interphase aggregated later than cells exposed continuously; also, washing and resuspending cells in buffer (the usual procedure used to start development in a population) provides initial conditions with low c-AMP concentration which, on this hypothesis, should be as effective as the addition of PDE. We conclude that the acceleration of the development of aggregation competence by extracellular PDE is probably a continuous process in which development takes place more quickly when c-AMP concentrations are kept low; however, our experiments do not distinguish between complementary models in which either high c-AMP concentration inhibits development or low c-AMP accelerates it.

Gerisch & Hess (1974) and Roos *et al.* (1975) showed that spontaneous oscillations of extracellular c-AMP may be induced or synchronized at early stages of interphase (t_3) with artificial pulses of c-AMP. Gerisch *et al.* (1975a) observed that expression of membrane-bound PDE, c-AMP receptor sites and contact sites A was accelerated by artificial pulses of c-AMP. Also Darmon *et al.* (1975) found that aggregation competence developed in AX2 cells in 4 h when they were continuously signalled with c-AMP pulses of 10^{-6} to 10^{-8} M for 3 h; this compares with a t_{agg} of 6 h in controls. Thus, there is strong evidence that oscillations in c-AMP synthesis, released or ejection, control the rate of development. It is also known (Gerisch & Hess, 1974) that a constant flow of 3 to 50 nmol c-AMP/min/l suppresses pre-established oscillations in c-AMP secretion and probably also suppresses oscillations in intracellular c-AMP concentration. It seems that acceleration of development of aggregation competence could never be related to steady concentrations of c-AMP.

The mechanism of induction and suppression of c-AMP oscillations is not known. Since oscillations occur synchronously throughout the population (Gerisch & Hess, 1974), it seems that c-AMP receptors must somehow be involved. These receptors may be an integral part of the control loop generating c-AMP oscillations; alternatively, binding of

c-AMP to receptors may be translated into other signals which then affect the control loop (Gerisch *et al.*, 1975*b*). In either case there is clearly a possibility that c-AMP oscillations can be suppressed if c-AMP receptors are saturated by extracellular c-AMP. We think that the effects of PDE we have observed can be explained in this way. By hydrolysing extracellular c-AMP, PDE clears receptor sites and lowers the level of background noise against which c-AMP pulses must be detected. This allows c-AMP pulses to be detected early in interphase at times when the signals are probably too weak, and the efficiency of detection too low, to produce a response.

We found that PDE had a particularly marked effect on development during the first hour of interphase. Presumably, at this time suppression of c-AMP oscillations by extracellular c-AMP is maximal; this is reasonable, since one might expect that in the first hour c-AMP receptors are few (so efficiency of signal detection is poor) and endogenous PDE is low (so a high 'noise' level of extracellular c-AMP can accumulate) and it is known that c-AMP is secreted in early interphase (Malkinson & Ashworth, 1973).

It appears (Fig. 7) that development is affected by extracellular PDE from the beginning of interphase. This implies that the c-AMP oscillator is already able to function at t_0 and is therefore not dependent on the development occurring during interphase, in contrast to the observation of Darmon *et al.* (1975) that the first hour of artificial c-AMP signalling was ineffective in accelerating development. Possibly the lag, in their experiments, corresponded to a period when PDE activity was still too low to remove background noise efficiently; after this period, artificial signalling with c-AMP pulses (10^{-6} to 10^{-8} M) promoted an increased rate of differentiation. We interpret these results as indicating that the background noise at and after t_1 is smaller than 10^{-8} M-c-AMP and that natural signals have a smaller amplitude or frequency than the artificial signals.

The relationship between c-AMP oscillations and development in *D. discoideum* is currently a matter of great interest. Our study prompts us to ask how closely coupled these processes are: whether c-AMP oscillations are indispensable for development to aggregation competence, or whether they simply control the rate at which development progresses.

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REFERENCES

- ALCÂNTARA, F. & MONK, M. (1974). Signal propagation in the cellular slime mould *Dictyostelium discoideum*. *Journal of General Microbiology* **85**, 321–324.
- ASHWORTH, J. M. (1971). Cell development in the cellular slime mould *Dictyostelium discoideum*. *Symposia of the Society for Experimental Biology* **25**, pp. 27–49.
- BARKLEY, D. S. (1969). Adenosine-3'-5'-phosphate: identification as acrasin in a species of cellular slime mold. *Science, New York* **165**, 1133–1134.
- BEUG, H., KATZ, F. E. & GERISCH, G. (1973*a*). Dynamics of antigen membrane sites relating to cell aggregation in *Dictyostelium discoideum*. *Journal of Cell Biology* **56**, 647–658.
- BEUG, H., KATZ, F. E., STEIN, A. & GERISCH, G. (1973*b*). Quantitation of membrane sites in aggregating *Dictyostelium* cells by use of tritiated univalent antibody. *Proceedings of the National Academy of Sciences of the United States of America* **70**, 3150–3154.
- BONNER, J. T. (1970). Induction of stalk cell differentiation by cyclic AMP in the cellular slime mold *Dictyostelium discoideum*. *Proceedings of the National Academy of Sciences of the United States of America* **65**, 110–113.
- BONNER, J. T., BARKLEY, D. S., HALL, E. M., KONIJN, T. M., MASON, J. W., O'KEEFE, G. & WOLFE, P. B. (1969). Acrasin, acrasinase, and the sensitivity to acrasin in *Dictyostelium discoideum*. *Developmental Biology* **20**, 72–87.
- BONNER, J. T., KELSO, A. P. & GILLMOR, R. G. (1966). A new approach to the problem of aggregation in the cellular slime molds. *Biological Bulletin* **130**, 28–42.

- CHASSY, B. M. (1972). Cyclic nucleotide phosphodiesterase in *Dictyostelium discoideum*: interconversion of two enzyme systems. *Science, New York* **175**, 1016–1018.
- CHASSY, B. M., LOVE, L. L. & KRICHEVSKY, M. I. (1969). The acrasin activity of 3'-5'-cyclic nucleotides. *Proceedings of the National Academy of Sciences of the United States of America* **64**, 296–303.
- CHEUNG, W. Y. (1967). Properties of cyclic 3'-5'-nucleotide phosphodiesterase from rat brain. *Biochemistry* **6**, 1079–1087.
- DAVIS, B. J. (1964). Disc electrophoresis. II. Method and application to human serum proteins. *Annals of the New York Academy of Sciences* **121**, 404–427.
- DARMON, M., BRACHET, P. & PEREIRA DA SILVA, L. H. (1975). Chemotactic signals induce cell differentiation in *Dictyostelium discoideum*. *Proceedings of the National Academy of Sciences of the United States of America* **72**, 3163–3166.
- GERISCH, G. (1965). Stadienspezifische Aggregationsmuster bei *Dictyostelium discoideum*. *Roux Archiv für Entwicklungsmechanik der Organismen* **156**, 127–144.
- GERISCH, G. (1968). Cell aggregation and differentiation in *Dictyostelium*. In *Current topics in Developmental Biology*, vol. 3, pp. 157–197. Edited by A. A. Moscona and A. Monroy. New York and London: Academic Press.
- GERISCH, G., FROMM, H., HUESGEN, A. & WICK, U. (1975a). Control of cell-contact sites by cyclic-AMP pulses in differentiating *Dictyostelium* cells. *Nature, London* **255**, 547–549.
- GERISCH, G. & HESS, B. (1974). Cyclic-AMP-controlled oscillations in suspended *Dictyostelium* cells: their relation to morphogenetic cell interactions. *Proceedings of the National Academy of Sciences of the United States of America* **71**, 2118–2122.
- GERISCH, G., HÜLSEN, D., MALCHOW, D. & WICK, U. (1975b). Cell communication by periodic cyclic-AMP pulses. *Philosophical Transactions of the Royal Society B* **272**, 181–192.
- GERISCH, G. & MALCHOW, D. (1974). Cell communication and cyclic-AMP regulation during aggregation of the slime mold *Dictyostelium discoideum*. In *Biochemistry of Sensory Functions*, pp. 279–298. Edited by L. Jaenicke. Berlin, Heidelberg and New York: Springer-Verlag.
- GERISCH, G., MALCHOW, D., RIEDEL, V., MÜLLER, E. & EVERY, M. (1972). Cyclic AMP phosphodiesterase and its inhibitor in slime mould development. *Nature New Biology* **235**, 90–92.
- GOIDL, E. A., CHASSY, B. M., LOVE, L. L. & KRICHEVSKY, M. I. (1972). Inhibition of aggregation and differentiation of *Dictyostelium discoideum* by antibodies against adenosine 3'-5'-cyclic monophosphate diesterase. *Proceedings of the National Academy of Sciences of the United States of America* **69**, 1128–1130.
- KONIUN, T. M. (1968). Chemotaxis in the cellular slime moulds. II. The effect of cell density. *Biological Bulletin* **134**, 298–304.
- KONIUN, T. M. & RAPER, K. B. (1961). Cell aggregation in *Dictyostelium discoideum*. *Developmental Biology* **3**, 725–756.
- KONIUN, T. M., BARKLEY, D. S., CHANG, Y. Y. & BONNER, J. T. (1968). Cyclic AMP: a naturally occurring acrasin in the cellular slime moulds. *American Naturalist* **102**, 225–233.
- LIN, Y. M., LIU, Y. P. & CHEUNG, W. Y. (1974). Cyclic 3'-5'-nucleotide phosphodiesterase. Purification, characterisation and active form of the protein activator from bovine brain. *Journal of Biological Chemistry* **249**, 4943–4954.
- LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. & RANDALL, R. J. (1951). Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry* **193**, 265–275.
- MALCHOW, D., FUCHILA, J. & NANJUNDIAH, V. (1975). A plausible role for a membrane-bound cyclic AMP phosphodiesterase in cellular slime mold chemotaxis. *Biochimica et biophysica acta* **385**, 421–428.
- MALCHOW, D. & GERISCH, G. (1974). Short-term binding and hydrolysis of cyclic 3'-5'-adenosine monophosphate by aggregating *Dictyostelium* cells. *Proceedings of the National Academy of Sciences of the United States of America* **71**, 2423–2427.
- MALCHOW, D., NÄGELE, B., SCHWARZ, H. & GERISCH, G. (1972). Membrane-bound cyclic AMP phosphodiesterase in chemotactically responding cells of *Dictyostelium discoideum*. *European Journal of Biochemistry* **28**, 136–142.
- MALKINSON, A. M. & ASHWORTH, J. M. (1973). Adenosine 3'-5'-cyclic monophosphate concentrations and phosphodiesterase activities during axenic growth and differentiation of cells of the cellular slime mould *Dictyostelium discoideum*. *Biochemical Journal* **134**, 311–319.
- MICHAL, G. & BERGMAYER, H. V. (1970). In *Methoden der Enzymatischen Analyse*, 2nd edn, p. 2060. Edited by H. V. Bergmeyer. Weinheim: Verlag Chemie.
- NEWELL, P. C. (1971). The development of the cellular slime mould *Dictyostelium discoideum*: a model system for the study of cellular differentiation. *Essays in Biochemistry* **7**, 87–126.
- RAPER, K. B. (1950). Pseudoplasmodium formation and organisation in *Dictyostelium discoideum*. *Journal of the Elisha Mitchell Scientific Society* **56**, 241–282.
- RIEDEL, V. & GERISCH, G. (1971). Regulation of extracellular cyclic-AMP-phosphodiesterase activity during development of *Dictyostelium discoideum*. *Biochemical and Biophysical Research Communications* **42**, 119–124.
- RIEDEL, V., GERISCH, G., MÜLLER, E. & BEUG, H. (1973). Defective cyclic adenosine-3'-5'-phosphate-phosphodiesterase regulation in morphogenetic mutants of *Dictyostelium discoideum*. *Journal of Molecular Biology* **74**, 573–585.

- RIEDEL, V., MALCHOW, D., GERISCH, G. & NÄGELE, B. (1972). Cyclic AMP phosphodiesterase interaction with its inhibitor of the slime mould *Dictyostelium discoideum*. *Biochemical and Biophysical Research Communications* **46**, 279–287.
- ROBERTSON, A., DRAGE, D. J. & COHEN, M. H. (1972). Control of aggregation in *Dictyostelium discoideum* by an external periodic pulse of cyclic adenosine monophosphate. *Science, New York* **175**, 333–335.
- ROOS, W., NANJUNDIAH, V., MALCHOW, D. & GERISCH, G. (1975). Amplification of cyclic-AMP signals in aggregating cells of *Dictyostelium discoideum*. *FEBS Letters* **53**, 139–142.
- SHAFFER, B. M. (1956). Acrasin, the chemotactic agent in cellular slime moulds. *Journal of Experimental Biology* **33**, 645–657.
- SHAFFER, B. M. (1957). Aspects of aggregation in cellular slime moulds. I. Orientation and chemotaxis. *American Naturalist* **91**, 19–35.
- THOMPSON, W. J. & APPLEMAN, M. M. (1971). Multiple cyclic nucleotide phosphodiesterase activities from rat brain. *Biochemistry* **10**, 311–316.
- WATTS, D. J. & ASHWORTH, J. M. (1970). Growth of myxamoeba of the cellular slime mould *Dictyostelium discoideum* in axenic culture. *Biochemical Journal* **119**, 171–174.